PATENT

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In re:	Patent application of Graham McCreath, *et al.*	:	
Serial No.	09/814,371	:	Group Art Unit: 1651
Filing Date:	March 22, 2001	:	Examiner: Susan Marie Hanley
For:	PURIFICATION OF FIBRINOGEN FROM FLUIDS BY PRECIPITATION AND HYDROPHOBIC CHROMATOGRAPHY	:	Conf. No. 4297

DECLARATION OF HENRICUS A. VAN VEEN UNDER 37 C.F.R. 1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, Henricus A. van Veen, declare as follows.

1. I am a Senior Process Development scientist in the Research and Development Department of Pharming Technologies B.V., Leiden, Netherlands, an affiliate of the owner of the present patent application. My *curriculum vitae* is attached.

CERTIFICATE OF MAILING UNDER 37 C.F.R. 1.8(a)

I hereby certify that this paper, along with any paper referred to as being attached or enclosed, is being deposited with the United States Postal Service on the date indicated below, with sufficient postage, as first class mail, in an envelope addressed to: Mail Stop Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

BY Ms. Dawn R. Collins

DATE: August 18, 2006

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2. I have extensive experience in the development and application of protein purifications processes. I am well versed in various purification techniques such as precipitation, filtration, and chromatography. I have experience in the purification of biopharmaceuticals from transgenic animal milk. I have experience with the development of analytical assays used for protein purification and fractionation.

3. I have read and understand the office action dated April 19, 2006. The experiments described herein were conducted by me. Copies of the literature references mentioned in this declaration are attached.

4. Human fibrinogen is present in three naturally occurring forms which differ in the degradation of the Aα-chain: non-degraded fibrinogen (HMW, 340 kDa, ~70%); fibrinogen degraded at one Aα-chain (LMW, 300 kDa, ~30%); and fibrinogen degraded at both Aα-chains (LMW', 280 kDa, ~5%). Holm B & Godal HC (1984), "Quantitation of the three normally occurring plasma fibrinogens in health and during so-called 'acute phase' by SDS electrophoresis of fibrin obtained from EDTA-plasma", *Thromb. Res.* 35, 279-90. The physiological process causing the degradation of the Aα-chain is as yet unknown. Henschen AH (1993) "Human fibrinogen –structural variants and functional sites", *Thromb. Haemost.* 70, 42-7. Nevertheless, human fibrinogen purified from fresh plasma of healthy volunteers consists of these three forms which can be visualized by non-reduced SDS-PAGE. Holm B & Godal HC, *supra;* Kuyas C, Haeberli A, Walder P & Straub PW (1990) "Isolation of human fibrinogen and its derivatives by affinity chromatography on Gly-Pro-Arg-Pro-Lys-Fractogel", *Thromb. Haemost.* 63, 439-44. On reduced SDS-PAGE, fibrinogen purified from fresh plasma provides Aα, Bβ and γ bands in a ratio of about 1:1:1. Kuyas C, Haeberli A, Walder P & Straub PW (1990), "Isolation of human fibrinogen and its derivatives by affinity chromatography on Gly-Pro-Arg-Pro-Lys-Fractogel", *Thromb. Haemost.* 63, 439-44; Longas MO, Newman J & Johnson AJ (1980) "An improved method for the purification of human fibrinogen", *Int. J. Biochem.* 11, 559-64.

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5. Taken together, these observations from the literature indicate that reduced SDS-PAGE of freshly purified human fibrinogen does not discriminate between the three naturally occurring forms of fibrinogen, whereas non-reduced SDS-PAGE does.

6. I have reviewed the German Patent 4240119 of Jennissen *et al.* Jennissen shows, on reduced SDS-PAGE, the recovery of the three chains of fibrinogen from plasma in a ratio of Aα, Bβ and γ of 1:1:1 (see column 5, lines 43-49 of the original German document, and page 6, lines 19-22 of the English translation). Since reduced SDS-PAGE cannot discriminate between the three naturally occurring variants of the fibrinogen Aα-chain, the results of Jennissen do not indicate separation of the naturally occurring Aα-chain degraded forms. Further, Figure 1 of Jennissen shows an almost complete binding of fibrinogen from plasma to the pentyl Sepharose column, which was eluted in only one peak. No additional peaks containing fibrinogen are observed that might indicate separation of the naturally occurring Aα-chain degraded forms of fibrinogen. Therefore, Jennissen does not describe the elimination of these degraded forms to obtain high Aα-chain integrity fibrinogen.

7. It has been found that the ability of HIC to separate fibrinogen from its Aα-chain degraded forms depends on the source of the fibrinogen. Human fibrinogen obtained from fresh frozen plasma was subjected to HIC on butyl Sepharose. The conditions, and the results, are set forth in Attachment 1. The fibrinogen eluted as a single peak. This indicates that the naturally circulating human variant forms of the Aα-chain (HMW, LMW and LMW') were not separated by HIC.

8. Recombinant human fibrinogen from transgenic bovine milk was subjected to HIC on butyl Sepharose, under the same conditions. The results are set forth in Attachment 2. In contrast to the single peak obtained from chromatography of human plasma fibrinogen, purified recombinant human fibrinogen from transgenic bovine milk eluted in three peaks, the first of which represents the elution of high Aα-chain integrity fibrinogen (HMW fibrinogen).

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

August 01, 2006
(date)

HENRICUS A. VAN VEEN

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AN IMPROVED METHOD FOR THE PURIFICATION OF HUMAN FIBRINOGEN

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(*Received* 29 *October* 1979)

Abstract—1. The isolation of human fibrinogen by differential polyethylene glycol precipitations of the fibrinogen contained in the cryoprecipitate of plasma is described.
2. Purification was performed in the presence of 1 mM diisopropyl fluorophosphate and trasylol.
3. The protein obtained is >95% clottable.
4. It contains no detectable prothrombin, thrombin, plasminogen or plasmin.
5. It is highly soluble and apparently undegraded.

INTRODUCTION

Fibrinogen, a plasma protein synthesized in the liver (Doolittle, 1973), is composed of three pairs of non-identical polypeptide chains (Aα, Bβ and γ) connected by disulfide bridges (Blombäck & Yamashima, 1958). Its molecular weight determined by sedimentation equilibrium is 340,000 ± 20,000 daltons (Caspary & Kekwick, 1957) distributed among its subunits as follows: 63,500, 56,000 and 47,000 daltons for the Aα, Bβ and γ chains respectively (McKee *et al.*, 1966).

Partial proteolysis of fibrinogen by thrombin during the coagulation of blood produces fibrin, the precursor of the blood clot (Lundblad *et al.*, 1977). The significant role of fibrinogen in the coagulation–fibrinolytic system has stimulated extensive investigation of its physicochemical properties (Doolittle, 1973).

Numerous techniques have been reported for the purification of this protein (Doolittle, 1973). However, most of them are time consuming, and unless extreme precautions are employed, the final product may be significantly denatured or partially degraded by proteolytic enzymes (Bang *et al.*, 1963; Lipinski *et al.*, 1963; Mosesson *et al.*, 1972; Shainoff, 1963). We found that even the best methods yield a protein which is defective in one or more aspects. For example, the highly soluble fibrinogen of Mosesson & Sherry (1966) was not suitable for our purposes because of its glycine contamination, which we could not remove. Therefore, we devised a procedure that yields undegraded fibrinogen, free of exogenous amino acids. A preliminary form of the procedure was published previously Lackner *et al.*, 1970).

This communication describes the purification of human fibrinogen in the presence of 1 mM diisopropyl fluorophosphate (DFP) and trasylol, by a simple method which involves treatment of the cryoprecipitate of plasma with $Al(OH)_3$ gel, followed by two differential polyethylene glycol (PEG) precipitations.

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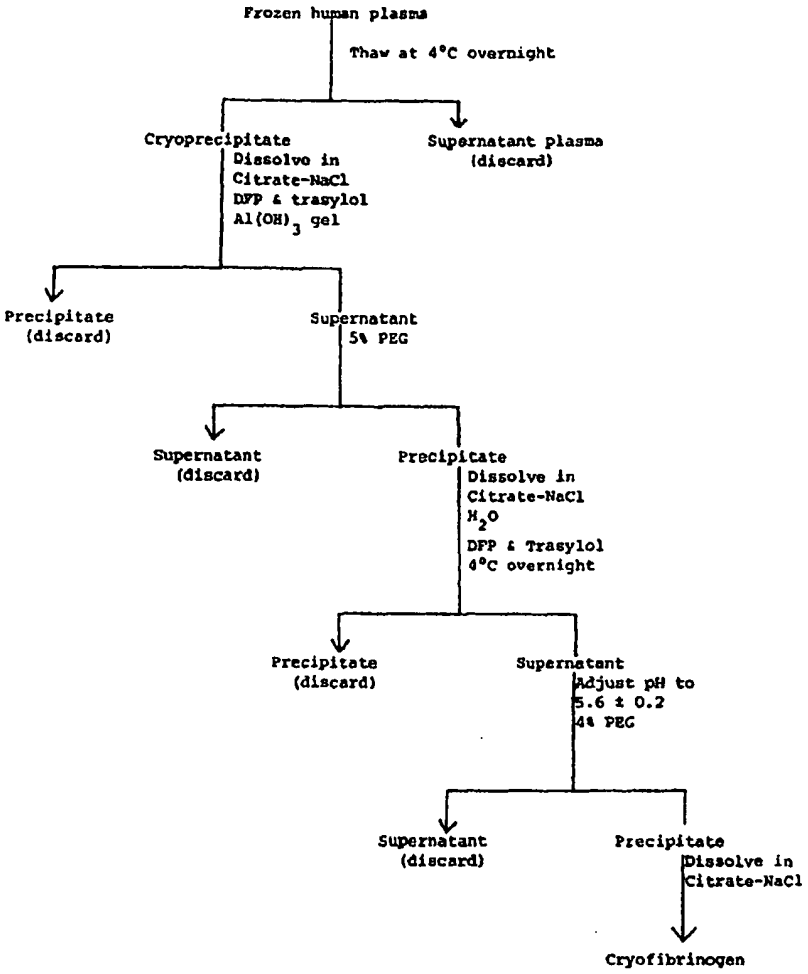
MATERIALS AND METHODS

Human thrombin (2500 NIH units/mg of protein, 87.5% α-thrombin) was a gift from Dr J. W. Fenton II, Division of Laboratories and Research, New York State, Department of Health, Albany, NY 12201. Ristocetin sulfate (90% Ristocetin A, Lyophilized) was from Lenau 98, Gl. Kongevej DK-1850 Copenhagen V Denmark. Human Platelets were a gift from Dr M. Zucker, New York University Medical Center. Trasylol 5000 KIU/ml was purchased from Mobay Chem. Co., New York, NY 10022. Citrated fresh frozen plasma was obtained from the blood bank at Memorial Hospital, New York, NY 10021 and stored at −80°C until used. Polyethylene glycol-4000 was a product of Union Carbide, New York, NY. Bovine serum albumin and sodium dodecylsulfate (SDS) were from Sigma Chem. Corp., St Louis, MO 63178. All other chemicals were of the best quality commercially available and were used without further purification.

Purification of fibrinogen

Unless otherwise indicated, the buffer used throughout this procedure was 0.15 M NaCl, 0.02 M sodium citrate, pH 7.4. Centrifugation was carried out at room temperature (22°C) and 3000 *g* for 10 min. The fibrinogen containing precipitates were washed with distilled ice-cold water. Thawing of plasma was initiated at 37°C (5–10 min) and continued at 4°C overnight (Ware *et al.*, 1947). During this process, the temperature of plasma was not allowed to rise above 0°C. Thus, centrifugation to remove the cryoprecipitate was performed when the ice had just melted, and the centrifuge temperature was 4°C. The cryoprecipitate was washed thoroughly and dissolved in a minimum volume of buffer at room temperature. This solution was adjusted to 1 mM in DFP and trasylol, mixed with 2% $Al(OH)_3$ gel (96:4, v/v), stirred at room temperature for 10 min, and the precipitate was removed by centrifugation. The supernatant was made 5% in PEG, at pH 7.4, stirred at room temperature for 30 min and centrifuged. The resulting precipitate was washed, dissolved in a minimum volume of buffer, mixed with an equal volume of distilled water and adjusted to 1 mM in DFP and trasylol. After incubation at 4°C overnight, a precipitate was removed by centrifugation (Mosesson & Sherry, 1966). The pH of the supernatant was adjusted to 5.6 ± 0.2 with 0.04 M citric acid, and fibrinogen was precipitated with 4% PEG as described above. The final product was washed, dissolved in a minimum volume of buffer, shell frozen and stored at −80°C or lyophilized. An outline of this procedure appears in Scheme 1.



Scheme 1. Purification of human fibrinogen.

Protein assay

The concentration of purified fibrinogen determined by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard or by absorbance at 280 nm, using 15.5 as the absorption coefficient ($E^{1\%}_{1cm}$) (Mosesson & Sherry, 1966).

RESULTS AND DISCUSSION

Different methods were used to evaluate the purity of fibrinogen (Table 1). The clottability was established by the method of Regoeczi (1967), as modified by Mosesson & Sherry (1966), using the following formula to determine the clottability:

$$\% \text{ clottability} = 100 - \frac{\text{O.D. 280 nm of clotted solution} - \text{O.D. 280 nm of blank}}{\text{O.D. 280 nm of unclotted solution}} \times 100$$

Immunoelectrophoresis of fibrinogen was performed on agarose plastic plates (Corning Acl) in 0.05 M sodium barbital, pH 8.6 at 10 V/cm for 35 min. Rabbit antihuman serum was added to the center well and allowed to diffuse for 36 hr at 4°C (Scheidegger, 1955).

Table 1. Human fibrinogen (purity evaluation)

Parameter Analyzed	Observations
Clottability with thrombin	>95%
Immunologic assay against rabbit antihuman serum	One precipitin line
vWF with platelets and Ristocetin	Traces
Plasminogen and plasmin	None
Prothrombin	None
Thrombin	None
Solubility in 0.15 M NaCl, 0.02 M sodium citrate or 0.3 M KCl	Readily soluble at room temperature
Stability: shell frozen at −80°C or lyophilized	Longer than 1½ years
NH_2-terminal amino acids	Alanine and tyrosine in a molar ratio of 1:1
Protein-staining on SDS polyacrylamide gels (Fig. 1)	Three major bands

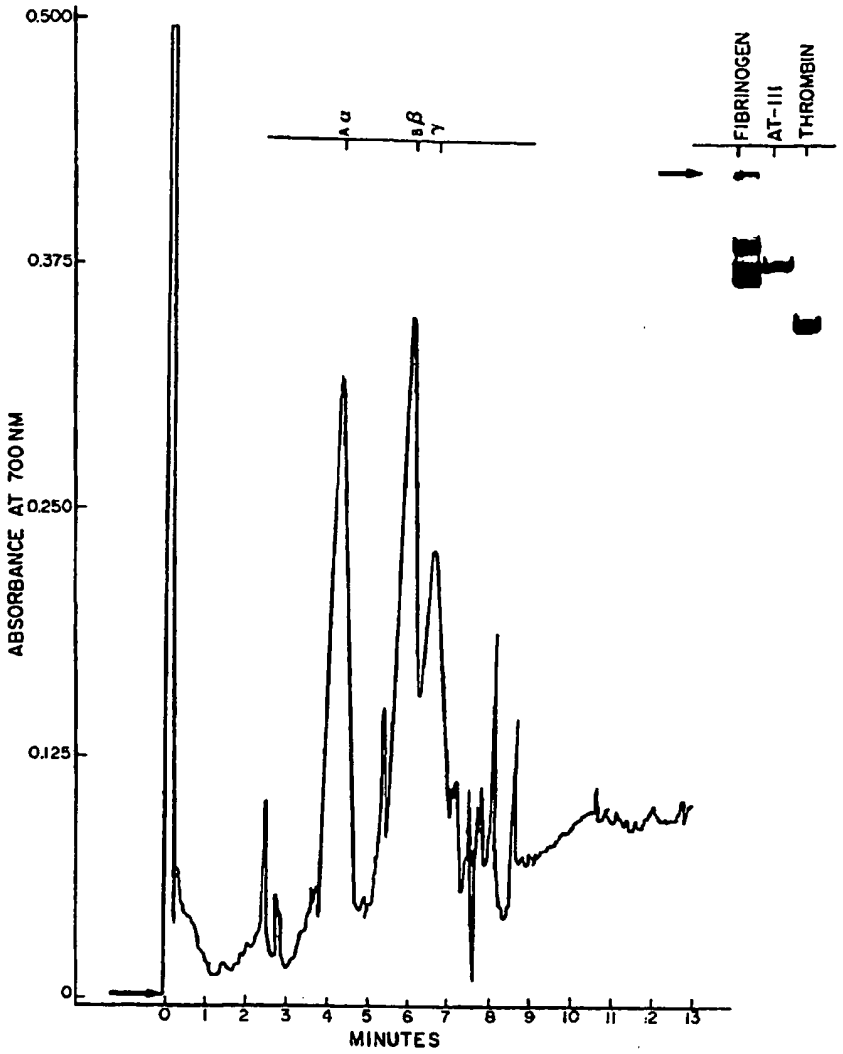


Fig. 1. Scan of a protein-stained 7.5% SDS polyacrylamide (slab) gel of human fibrinogen at 700 nm. The gel used in the scan appears in the upper right side. The arrows indicate the cathode of the gel and the beginning of the scan. Human antithrombin-III (mol wt 56,000 daltons) and thrombin (mol wt 32,000 daltons) were included as standards. The proteins were reduced with 1% 2-mercaptoethanol at 100°C for 2 min. Each band represents 8 μg of protein electrophoresed according to the method of Weber & Osborn (1969). Scanning was carried out in a Gilford spectrophotometer model 240 with Gilford recorder model 6051.

A modification of the method of Brinkhous *et al.* (1977) was used to detect von Willebrand factor (vWF) in fibrinogen. Platelets (200 μl, approx. $5 \times 10^5/mm^3$ in 0.05 M imidazole, 0.1 M NaCl, pH 7.2) and fibrinogen (250 μl, 10.0 mg/ml in 0.02 M sodium citrate, 0.15 M NaCl, pH 7.4) were mixed and incubated at 37°C for 1 min with constant shaking. Ristocetin (50 μl, 10.0 mg/ml) was added, and shaking continued for 3 min. Agglutination was determined by visual comparison of the fibrinogen containing sample with the agglutination of normal human plasma at different dilutions. Only traces of vWF were detected by this method.

The presence of plasminogen was tested as follows: fibrinogen (0.9 ml, 5 mg/ml in 0.05 M Tris, 0.15 M NaCl, pH 8.3) and streptokinase (10,000 units/ml) were incubated at 37°C for 1 hr. Aliquots of this solution (100–500 μl) were combined with 100 μl of 0.5 mM CBZ-Gly-Pro-Arg-*p*-nitroanilide in a total volume of 600 μl adjusted with 0.05 M Tris, 0.15 M NaCl, pH 8.3 and maintained at 37°C. Plasmin activity was monitored at 405 nm (Svendsen *et al.*, 1973) in a Gilford spectrophotometer model 240 equipped with a 6051 Gilford recorder. No activity was observed in the fibrinogen containing samples. Although standard plasminogen was efficiently converted to plasmin under the same conditions. Notice that fibrinogen preparations performed in the absence of trasylol contained traces of plasminogen, detectable by this method.

In order to determine if fibrinogen was contaminated with thrombin, the clotting assay of Lundblad *et al.* (1976) was employed, except that thrombin was excluded from the assay mixture. The resulting clotting time was > 120 min.

Polyacrylamide SDS-gel electrophoresis of fibrinogen after reduction with 1% 2-mercaptoethanol at 100°C for 2 min (Weber & Osborn, 1969) reveals three major bands with apparent molecular weights of 64,000, 56,000 and 47,000 daltons which represent the Aα, Bβ, and γ subunits reported by McKee *et al.* (1966). Absorbance of these (protein-stained) bands after scanning of the gel at 700 nm displays three peaks whose areas are in a ratio of 1:1:1 (Fig. 1). These data suggest that the protein was not degraded during purification (Pizzo *et al.*, 1972).

The NH_2-terminal amino acids determined by the method of Edman (1956), as modified by Irion & Blombäck (1970) are Alanine and Tyrosine in a molar ratio of 1:1. These results are in accord with the ones reported by Dayhoff (1972) and Blombäck & Yamashina (1958) and also indicate integrity of the fibrinogen obtained.

Table 1 shows that fibrinogen isolated by the method outlined in this communication is highly pure, stable, readily soluble in dilute salt solutions, undegraded and highly clottable.

The yield varied from 22 to 25%, assuming the concentration of fibrinogen in plasma to be 3 mg/ml. The method is quick; except for the two overnight incubations (Scheme 1), 2 litres of plasma may be processed in 6 hr. It also proved to be reproducible over a range of ten preparations.

Acknowledgements—This work was supported by NIH Grant HL 15596.

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Isolation of Human Fibrinogen and its Derivatives by Affinity Chromatography on Gly-Pro-Arg-Pro-Lys-Fractogel

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Summary

With an immobilized synthetic pentapeptide GlyProArgPro-Lys comprising the N-terminal sequence GlyProArg of the α-chain of fibrin, a new affinity method for the quantitative isolation of fibrinogen out of anticoagulated plasma was developed. The method proved to be superior to all known isolation methods in respect to ease of use and yield, since fibrinogen could be isolated in one step out of plasma with a recovery of more than 95% when compared to the immunologically measurable amounts of fibrinogen. Moreover the amounts of contaminating proteins such as fibronectin, factor XIII or plasminogen were negligible and the purity of the isolated fibrinogen was higher than 95% as measured by polyacrylamide gel electrophoresis. The clottability was 90% and more. Another advantage of this affinity purification method is the possibility to isolate fibrinogen quantitatively out of small plasma samples (<5 ml). Further, abnormal fibrinogen molecules, provided their complementary binding site for GlyProArg is preserved, may also be quantitatively isolated independent of any solubility differences as compared to normal fibrinogen. In addition fibrin(ogen) fragments originating from plasmic digestion can be separated on the basis of their affinity to GlyProArg. The described affinity gel can be used more than 50 times without any loss of capacity.

Introduction

The isolation of human fibrinogen is generally carried out by plasma fractionation methods. Fibrinogen is precipitated from plasma either with ethanol (1), ammonium sulfate (2) or with β-alanine/glycine (3) with relatively high yield and homogeneity. All these fractionation methods require large amounts of plasma and the isolation of fibrinogen is by far not quantitative. The recovery of human fibrinogen out of plasma for all these methods is close to 60%. In addition some abnormal fibrinogens with a higher solubility (e.g. fibrinogen with increased sialic acid content) are recovered only to a reduced percentage, or even not at all.

Human fibrinogen has a strong affinity for fibrin. Therefore, fibrin immobilized on Sepharose is used to isolate fibrinogen from human plasma by affinity chromatography (4). We tried to simulate the affinity between fibrinogen and fibrin by immobilizing GlyProArgProLys to CDI-activated Fractogel, a solid hydrophylic polymer. Laudano and Doolittle have shown that the tetrapeptide GlyProArgPro, containing the N-terminal sequence of the α-chain of fibrin being exposed upon the action of thrombin on fibrinogen, competitively inhibits the fibrin polymerization. It also binds to fibrinogen (5, 15).

The N-terminal amino acid sequence GlyProArg is involved in the initiation of the fibrin polymerization by binding to the complementary binding site of an other fibrin(ogen) molecule. Based on this, a fast and quantitative method for the isolation of human fibrinogen from plasma by GlyProArgProLys affinity chromatography was developed. It was assumed that any fibrinogen molecule with an intact complementary binding site should bind to this gel.

Materials and Methods

Peptide Synthesis

Boc-amino acids were obtained from Bachem AG (Switzerland). All chemicals and solvents used were of highest purity commercially available.

Boc-^{14}C-glycine was obtained by reacting ^{14}C-glycine (Amersham, GB) with di-tert-butylcarbonate (Fluka, Switzerland) and was purified according to Moroder et al. (6). The peptide tBoc-GlyProArgProLys-O-methylester was synthesized with classical solution method. The lysine ε-amino- and the arginine guanido group was protected with a carbobenzoxy group (Z group). The coupling reactions were performed in methylene chloride with dicyclohexylcarbodiimide and 1-hydroxybenzotriazole according to König and Geiger (7). Boc was removed during the synthesis with 80% trifluoroacetic acid in water. By-products were extracted with acid and base washes. The Z-protecting groups were removed by catalytic hydrogenation with 10% Pd-charcoal (Fluka) in 90% aqueous acetic acid in a hydrogenation apparatus for 6 h at room temperature. After removal of charcoal by filtration, organic soluble by-products were extracted with diethylether and the peptide was obtained from the aqueous phase by lyophilization.

Boc-GlyProArgProLys-O-methylester was dissolved in water, and the amino acid composition and concentration was determined by amino acid analysis on a Liquimat (Kontron AG, Switzerland) after total acid hydrolysis (6 M HCl, 24 h, 108° C). An aliquot of the peptide stock solution was measured for its ^{14}C-glycine content to determine the specific activity of the tBoc-GlyProArgProLys-O-methylester.

The peptide was further analyzed by reversed phase chromatography on a PepRPC HR 5/5 column (Pharmacia, Sweden) with a FPLC-liquid chromatography system (Pharmacia, Uppsala, Sweden). Solvent A was 0.1% TFA/H_2O, solvent B was 0.1% TFA/acetonitrile (Fluka, HPLC grade). Separations were carried out with a linear gradient from 0–50% B in 30 min at a flow rate of 1 ml/min. The peptide peak was detected by monitoring the adsorption at 220 nm with a Kratos UV-detector (Kratos, USA).

Immobilization of tBoc-GlyProArgProLys to Fractogel

2 mMoles tBoc-GlyProArgProLys-O-methylester was dissolved in 40 ml 0.1 M Na_2CO_3, pH 9.5 and 10 g Fractogel TSK AF-CDI 650 (Merck, Darmstadt, FRG) was added to the peptide solution directly. The final concentration of the peptide in the coupling solution was 40 mM.

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The coupling was carried out at room temperature for 48 h on a rotatory plate. The gel was then washed on a sintered glass filter with carbonate buffer, H_2O, isopropanol and finally methanol each 500 ml. The gel was dried in a desiccator under vacuum. For the final deprotection of the immobilized peptide, the gel was suspended in 50 ml neat TFA and was incubated at room temperature for one hour. Occasionally the gel was stirred with a glass rod. TFA was removed with vacuum filtration. The GlyProArgProLys-Fractogel was washed with 500 ml 2.5% K_2CO_3 solution to neutralize the remaining TFA. The gel was further extensively washed with H_2O and methanol. The affinity gel was dried again in a desiccator under vacuum. An aliquot of the dried gel was weighed out and the ^{14}C-glycine content determined in order to calculate the final coupling efficiency of the peptide. To evaluate any unspecific binding Fractogel TSK AF-CDI 650 was taken through the same procedure as when the peptide gel was prepared with the only difference that the addition of the peptide methylester was left out. The reaction time and the washing steps were identical.

Capacity Determination of GlyProArgProLys-Fractogel for Fibrinogen

1 g of the affinity gel was suspended in 0.05 M triethanolamine, 0.1 M NaCl, 0.02 M ε-aminocaproic acid, pH 7.4 (further referred as TEA-buffer) and was filled into a 5 ml syringe. The volume of the swollen gel was 4 ml. Citrated plasma was run through the column at 6 ml/h and fractions of 1 ml were collected. 5 IU thrombin was added to each running through plasma fraction. As soon as in one fraction a visible clot was obtained, the adsorption of plasma was stopped and the gel was washed in the column with TEA-buffer until the absorbance at 280 nm was 0.02 (flow rate 40 ml/h). The fibrinogen was desorbed with TEA buffer containing 6 M urea. From the amount of isolated fibrinogen the capacity of the gel was calculated. The fibrinogen concentration was obtained by spectrophotometric readings at 280 nm, using an extinction coefficient of 15.1 ($E^{1\%}_{1cm}$).

Isolation of Human Fibrinogen by GlyProArgProLys-Fractogel

2 g of GlyProArgProLys-Fractogel were suspended in TEA-buffer and poured into a glass column (2 × 5 cm). The gel volume was 7.8 ml. 4–10 ml of plasma anticoagulated either with 0.102 M citrate or with 500 IU heparin and 500 IU aprotinin (further called citrated or heparin/aprotinin plasma) was run through the column at a flow rate of 6 ml/h. After the plasma was run through, the column was first washed with TEA-buffer containing 0.5% gelatin (3 column volumes). The flow rate was then increased to 40 ml/h and the gel was washed with at least 10 column volumes of TEA-buffer containing 1 M NaCl. The fibrinogen was eluted from the gel with 3 different solutions: TEA buffer containing 6 M urea; 0.1 M acetate buffer, pH 4.5, containing 3 M urea; and 0.1 M acetate buffer, pH 4.5, containing 2 M urea. The fibrinogen containing fractions as determined by absorbance at 280 nm were pooled and dialyzed extensively against 0.05 M Tris, 0.1 M NaCl, pH 7.4 in the cold. The isolated fibrinogen was analyzed by SDS-polyacrylamide gel electrophoresis on 3.5% (non reducing conditions) and on 7% gels (reducing conditions) according to Weber and Osborn (8).

The plasma fractions were analyzed after the column passage for any residual fibrinogen by immunodiffusion against a polyclonal commercial anti-fibrinogen antiserum (Behring, Marburg, FRG). An even more sensitive determination of any residual fibrinogen was applied to the running through plasma fractions by the radioimmunologic determination of fibrinopeptide A after the addition of an excess amount of thrombin (2 IU) to each fraction for 3 h.

The clottability of the isolated fibrinogen fractions were determined as follows: 5 IU of thrombin in 20 µl were added to 1 ml of fibrinogen solution (1 mg/ml) and incubated at 37° C for 1 h. The clot was extensively washed with physiologic saline and finally excess liquid was removed from the clot by squeezing in a silk cloth. The clot was then dissolved in 1 ml of 6 M buffered urea. The clottability was calculated in percent by comparing the optical density at 280 nm of the redisolved clot to the original fibrinogen solution.

The presence of contaminating proteins such as factor XIII, fibronectin and plasminogen was checked by immunodiffusion of the isolated fibrinogen samples against polyclonal antisera against the respective proteins and compared to Kabi- and Imco-fibrinogen, all at a concentration of 4 mg/ml (LC-Partigen for fibronectin, M-Partigen for plasminogen, immunodiffusion according to Ouchterlony for factor XIII-S (all Behring, Marburg, FRG).

Separation of Plasmin Digest of Fibrinogen on GlyProArgProLys-Fractogel

25 mg fibrinogen (Kabi, Sweden) was dissolved in 5 ml 0.05 M Tris, 0.1 M NaCl, pH 7.4, containing 2.5 mM $CaCl_2$, 5,000 U urokinase (Choay, France) was added to the fibrinogen solution in order to activate the small amounts of plasminogen adsorbed. The digestion was carried out at 37° C for 5 h and was terminated by adding 50 µl 10^{-2} M D-Phenylprolyl-arginyl-chloromethyl ketone (PPACK) solution. The fibrinogen lysate was run through the GlyProArgProLys-Fractogel column (7.8 ml gel volume) at a flow rate of 6 ml/h. The column was previously equilibrated with TEA-buffer containing 5 mM $CaCl_2$. The column was then washed with TEA-buffer, and the adsorbed protein was eluted with TEA-buffer containing 6 M urea.

Another sample of fibrinogen was digested in the same way but in the presence of 10 mM EGTA in order to obtain a fibrinogen digest in the absence of calcium (9, 10). The EGTA-fibrinogen lysate was also applied to the affinity column under the same experimental conditions as with the lysate in presence of Ca^{2+}. The protein containing fractions were determined by absorbance measurements at 280 nm, pooled and dialyzed against TBS in the cold.

The non adsorbed and the adsorbed lysate fractions were analyzed by immunodiffusion against a polyclonal anti-D and anti-E antiserum (Behring, Marburg, FRG). The affinity isolated lysate fractions were further characterized by SDS-polyacrylamide electrophoresis on 7% gels.

Results

Reversed phase chromatography of the synthesized peptide showed that the purity was greater than 90%. The remaining 10% impurities were removed by preparative HPLC. The amino acid analysis of the t-BOC-GlyProArgProLys-O-methylester after acid hydrolysis gave the expected molar ratios of the amino acids contained in the peptide. After coupling of the peptide to CDI-Fractogel and final deprotection of the immobilized peptide the degree of substitution was calculated on the basis of the specific activity ^{14}C-glycine/peptide. The substitution of two different batches of gel prepared was 3.4 and 3.7 µmole peptide/g dry gel. The capacity of the GlyProArgProLys-Fractogel varied from 8–10 mg fibrinogen/ml wet gel depending on the preparation.

Human fibrinogen was almost quantitatively isolated from plasma by GlyProArgProLys-Fractogel affinity chromatography (Figs. 1 and 2). The plasma fractions running through the affinity column did not contain any immunologically detectable fibrinogen (detection limit below 25 µg/ml fibrinogen). Even the addition of thrombin to the plasma fractions after passage through the column did not give rise to the release of significant amounts of fibrinopeptide A, thus demonstrating an almost complete absence of fibrinogen. Fibronectin, a common contaminant of fibrinogen preparations, could be successfully eliminated by washing the affinity column with 0.5% gelatin prior to the elution of fibrinogen from the column. Also factor XIII and plasminogen were not detectable by immunodiffusion and the maximal possible contamination by these proteins of the fibrinogen was calculated to be lower than 0.5%. Both, Kabi- and Imco-fibrinogen gave a positive reaction for fibronectin and factor XIII.

The isolated fibrinogen (Fig. 2) was heterogeneous in respect to the molecular weight as determined by SDS-polyacrylamide gel electrophoresis. Three different populations were observed as they have already been described by several authors (e.g. 11, 12). Fibrinogen with a molecular weight of 340,000, representing the intact molecule, was the predominant form. Ten different fibrinogen preparations were separated electrophoretically on 3.5% polyacrylamide gels and the Coomassie blue stained gels were scanned at 545 nm. The mean relative proportions of the three

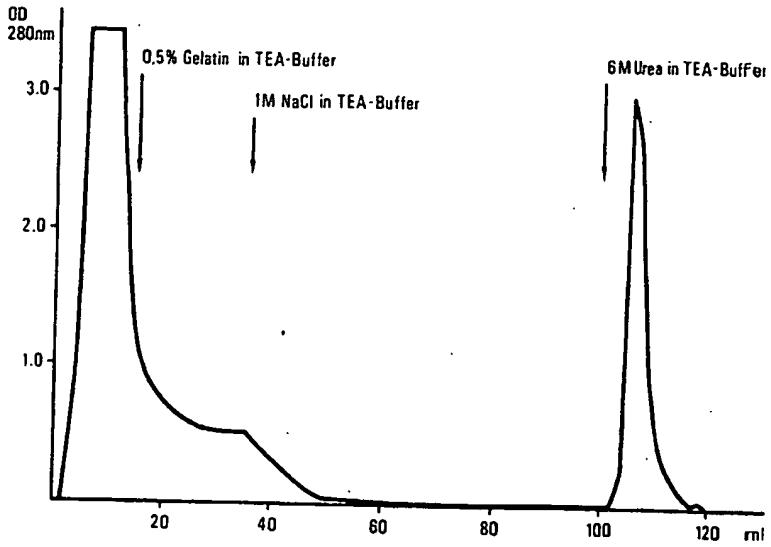

Fig. 1 Elution pattern of normal human plasma on GlyProArgProLys-Fractogel. The buffer changes are indicated by arrows. Fibrinogen was eluted either with 6 M urea (shown here) or with acid buffer containing 2 M urea (compare Material and Methods)

Table 1 Clottability of the GlyProArgProLys-Fractogel isolated fibrinogen in presence and absence of factor XIII

	Clottability (%), mean, range, n = 6 without factor XIII	with factor XIII (3 U/ml)
Kabi-Fibrinogen	92 (89–96)	95 (91–99)
Fibrinogen 1*	78 (65–87)	94 (89–100)
Fibrinogen 2**	80 (72–88)	96 (91–100)
Fibrinogen 3***	92 (90–96)	95 (92–98)

* Fibrinogen isolated by GlyProArgProLys-Fractogel affinity chromatography, desorption with 6 M urea, TEA buffer, pH 7.4.
** Fibrinogen isolated by GlyProArgProLys-Fractogel affinity chromatography, desorption with 3 M urea in 0.1 M acetate buffer, pH 4.5.
*** Fibrinogen isolated by GlyProArgProLys-Fractogel affinity chromatography, desorption with 2 M urea in 0.1 M acetate buffer, pH 4.5.

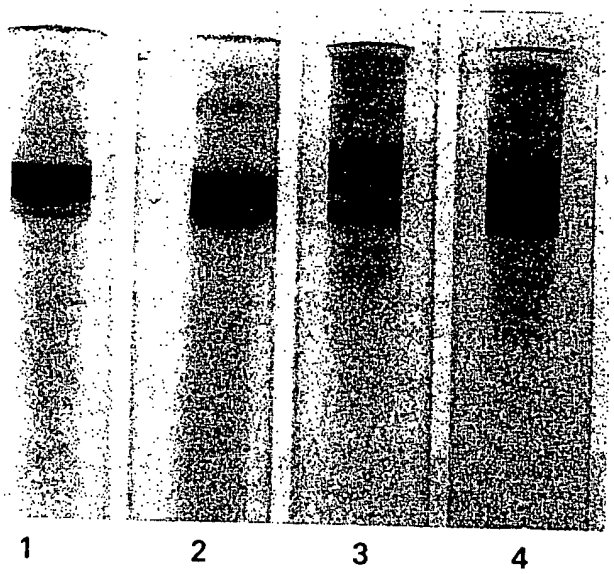

Fig. 2 Polyacrylamide gel electrophoresis of fibrinogen under non-reducing (3.5%) and reducing (7%) conditions in presence of sodium dodecyl sulfate. Reduction was performed with dithiothreitol. 1: GlyProArgProLys-Fractogel isolated fibrinogen, non-reduced. 2: Kabi Fibrinogen, non-reduced as control. 3: GlyProArgProLys-Fractogel isolated fibrinogen, reduced. 4: Kabi Fibrinogen, reduced as control

molecular forms of fibrinogen were 73%, 23% and 4% and in accordance with results recently reported for fibrin (12). The two minor fractions most probably represent two catabolic intermediates of fibrinogen (approx. mw 300,000 and 275,000). Since the blood was collected immediately into an anticoagulant solution containing heparin and aprotinin, conditions to completely inhibit any plasmin activity, we conclude that the isolated fibrinogen populations represent in vivo circulating forms of the molecule. This was the case for fibrinogen isolated from single donor or from pooled plasma.

When fibrinogen was eluted with 6 M urea at neutral pH, the clottability of the isolated fibrinogen was low with a mean of 78% and a range of 65% to 87%. The desorption with 3 M urea in acetate buffer, pH 4.5 gave identical results in respect to the completeness of the desorption, but the clottability could be slightly increased (compare Table 1). By lowering the urea concentration from 3 M to 2 M, again in acetate buffer, pH 4.5, the efficiency of desorption of fibrinogen could be maintained at 95% and higher and the clottability of the isolated fibrinogen was consistently higher and most of the times more than 90% (comparison of 2 M to 3 M urea elution in six single donor plasma).

The addition of activated factor XIII did increase the clottability of the fibrinogen samples isolated either by 6 M urea or 3 M urea in acetate buffer to some extent (compare Table 1). The addition of calcium up to 2.5 mmole/l did not increase the clottability of the fibrinogen samples desorbed with 6 M urea or 3 M urea in acetate buffer.

The specificity of the GlyProArgProLys-Fractogel was tested by displacing the bound fibrinogen with the free peptide GlyProArgPro in TEA-buffer (10^{-3} mole/l peptide). The fibrinogen was eluted in a larger volume than with the urea containing solutions, indicating that the binding of fibrinogen to the immobilized peptide was slightly stronger than to the free peptide. The final recovery was only slightly lower than with the urea containing solutions. Therefore specific displacement with the peptide GlyProArgPro is possible.

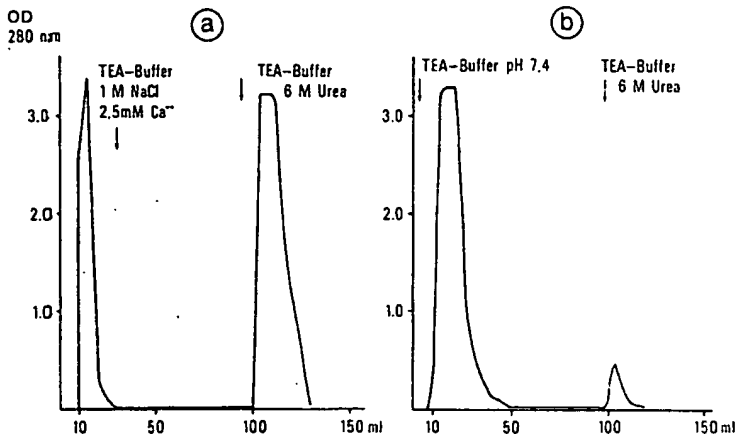

Fig. 3 Elution pattern on GlyProArgProLys-Fractogel of plasmin lysates of Imco-fibrinogen prepared and run in presence (a) or absence (b) of calcium, complexed by EGTA

The specificity of the GlyProArgProLys-Fractogel was further analyzed by chromatography of plasmic digests of fibrinogen obtained in the presence and absence of Ca^{2+}-ions. The elution patterns of these plasmic fragments are presented in Fig. 3. The run through fractions of the plasmic digest obtained in the presence of calcium contained fragment E and the C-terminal extensions of the α-chain, a fragment which is cleaved at early stages of the plasmin digestion of fibrin(ogen). Immunodiffusion of these fractions against anti-E- and anti-D-antisera gave a precipitation line only with anti-E-antibodies, showing that fragment D_1 is selectively and quantitatively removed from the lysate by the GlyProArgProLys-column. Fragment D_1 was eluted with 6 M urea with the same efficiency as intact fibrinogen. After removal of the urea by dialysis the fragment D_1 could again be adsorbed on the affinity column and then reeluted with urea. This indicates that the denaturing conditions (6 M urea) were not efficient enough to destroy the structure of the binding site, or most probably that renaturation of the binding site after removal of the urea is possible. Identical results were obtained when 6 M urea for desorption was substituted by 2 M urea in acetate buffer, pH 4.5.

When fibrinogen was digested in presence of EGTA, the resulting fragments D_2, D_3 and E were not retained by the GlyProArgProLys-Fractogel, and were eluted in the run through fractions (Fig. 4). The minor peak visible on the chromatogram (Fig. 3), was undigested D_1 fragment as verified by polyacrylamide gel electrophoresis in presence of SDS. This shows that even very small amounts of D_1 fragment or probably any fragment which contains the polymerization site may be isolated using GlyProArgProLys-Fractogel. In preliminary experiments we also

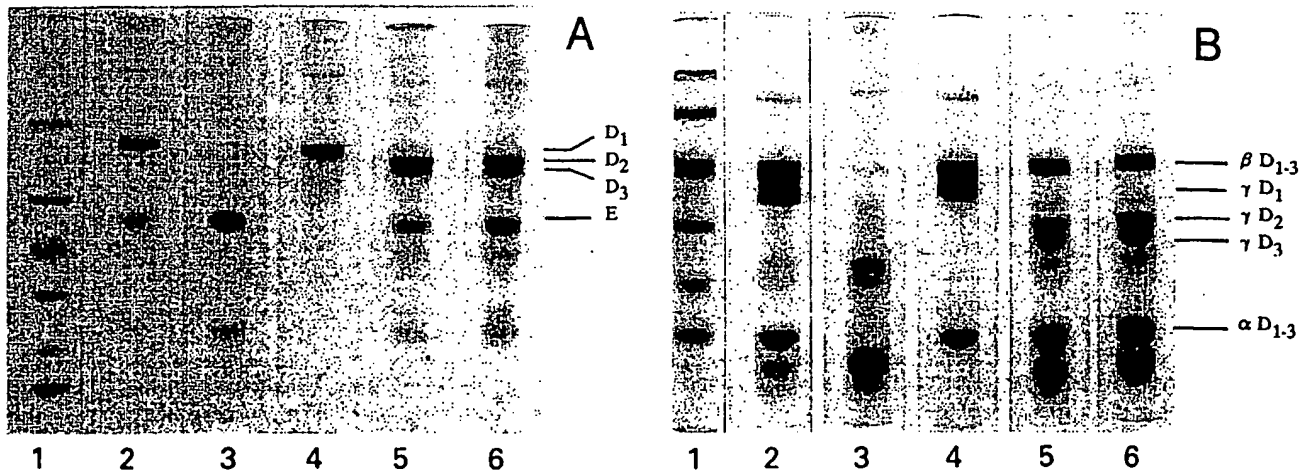

Fig. 4 Polyacrylamide gel electrophoresis of plasmin digests of Imco-fibrinogen and of the GlyProArgProLys-Fractogel isolated fractions in presence of sodium dodecyl sulfate under non-reducing (A) and reducing conditions with dithiothreitol (B). 1: molecular weight standards (from top: phosphorylase b, mw 94,000; albumin, mw 67,000; ovalbumin, mw 43,000; carbonic anhydrase, mw 30,000; trypsin inhibitor, mw 20,100; α-lactabumin, mw 14,400). Gels 2, 3 and 4 demonstrate the behavior of the fibrinogen degradation products produced by plasmin in the presence of calcium (2.5 mM): 2: complete plasmic digest in presence of calcium, before application to the GlyProArgProLys-Fractogel column. 3: run through fraction from the GlyProArgProLys-Fractogel column containing only fragment E and α-chain extension. 4: urea-eluted fraction from the GlyProArgProLys-Fractogel column containing fragment D_1. Gels 5 and 6 demonstrate the behavior of the fibrinogen degradation products produced by plasmin in the absence of calcium (10 mM EGTA): 5: complete plasmic digest in absence of calcium, before application to the column. The small amount of fragment D_1 present in this digest (compare Fig. 3b) is not visible on the gel. 6: run through fraction from the GlyProArgProLys-Fractogel column containing fragments D_2, D_3, E and α-chain extensions

tried to digest fibrinogen with other enzymes such as endoproteinase Arg C and Clostripain, but we could not isolate any other fragments except D_1.

For the evaluation of unspecific (non-peptide dependent) binding of fibrinogen to the matrix of the gel, a "blank" Fractogel column was prepared as described under "Material and Methods". Fibrinogen or citrated plasma was run through this column under identical conditions as with the peptide column. Fibrinogen did bind considerably to the matrix up to 4 mg per ml of wet gel. The binding, however, was much weaker, since 80% of the absorbed fibrinogen could be eluted with 1 M urea in TEA buffer (compared to less than 2% on the Gly-Pro-Arg-Pro-Lys-Fractogel column) and the remaining 20% were desorbed by 2 M urea in TEA buffer. The acid pH buffer was not tested because of the weak binding. Furthermore the purity of the desorbed fibrinogen isolated from plasma was lower than with the peptide column and estimated to be 80–85%.

Discussion

The isolation procedure described here demonstrates that human fibrinogen and its catabolic derivatives which contain the fibrin polymerization site, may easily be isolated from plasma using a single step affinity chromatography with GlyProArg-ProLys-Fractogel.

When this method is compared to the fibrin-affinity chromatography some practical advantages in the every day use of the GlyProArgProLys-Fractogel can be demonstrated. The interaction of immobilized fibrin with other plasma proteins with an affinity for fibrin (e.g. t-PA, thrombin) is eliminated by the use of this synthetic peptide. In our hands the fibrin-affinity column could only be used for 2 or 3 isolations of fibrinogen, since its capacity was progressively decreasing due to losses of fibrin. In contrast the GlyProArgProLys-Fractogel already used for more than 50 isolations is still performing well.

All fibrinogen preparations were soluble after removal of the urea by dialysis and all fibrinogen samples could be thawed and frozen several times without any loss of solubility. Although unspecific binding of fibrinogen was observed with the "blank" column, this did not challenge the general performance of the GlyProArgProLys-Fractogel column. The unspecific binding to the gel was considerably weaker since fibrinogen could quantitatively be eluted from the blank column with 2 M urea at neutral pH, whereas from the peptide column only trace amounts of fibrinogen (<5%) were desorbed under these conditions. It is suspected that the binding of fibrinogen is more likely due to some imidazole groups resulting from the non-reacting N,N-carbonyl-diimidazole groups than to interaction with the gel matrix itself.

The important native amino acid sequence present in the N-terminal part of the α-chain of fibrin is GlyProArg. Laudano and Doolittle have shown that the addition of a second proline enhances the affinity of the peptide GlyProArgPro for fibrinogen almost tenfold (5). To have the peptide distant enough from the gel matrix a further amino acid, lysine, was included in the peptide as a spacer. The carboxyl group of lysine not involved in the coupling reaction to carbodiimide activated Fractogel was protected by esterification in order to avoid any unspecific adsorption of other proteins on the basis of ionic interactions. Fractogel TSK (Merck, Darmstadt, FRG) was chosen as insoluble matrix because of its high exclusion limit of more than 500,000 Daltons and for its chemical and mechanical stability.

The interaction with the GlyProArgProLys-Fractogel is most likely specific for the complementary polymerization site. Thus, fragment D_2, lacking the last 55 C-terminal amino acids, and fragment D_3, lacking the last 109 C-terminal amino acids of the γ-chain did not bind to the GlyProArgProLys-Fractogel column. This is in agreement with the proposal made by several authors that the complementary fibrin polymerization site may be located in the C-terminal portion of the γ-chain (13, 14), and is further supported by the observation that when fragment D_1 is further degraded by plasmin in the absence of calcium to fragment D_2 or D_3, the anticlotting properties of fragment D_1 are lost (10). Our chromatographic experiments show that for the binding of fibrinogen or its derivatives to GlyProArgProLys an intact C-terminal γ-chain remnant of the fragment D may play an important role.

The ideal desorption method is certainly to displace the fibrinogen from the affinity column with the tetrapeptide Gly-ProArgPro, since in this way any denaturing condition can be avoided. It has been tested and it works almost as well as the other eluants used. But the use of the tetrapeptide GlyProArgPro to elute fibrinogen from the affinity column has two disadvantages. First, the tetrapeptide has to be used at 10^{-3} mole/l concentration to elute the fibrinogen in acceptably small volume. Therefore it is quite an expensive way to desorb fibrinogen. In addition, when fibrinogen is eluted with GlyProArgPro, the free peptide is bound to it, and has to be removed by extensive dialysis before any functional studies can be performed. Therefore, the desorption of fibrinogen from the GlyProArgProLys-Fractogel with the tetrapeptide GlyProArgPro may only be applicable in very special circumstances.

The elution system first used in our laboratory, 6 M urea in TEA buffer at neutral pH, may cause irreversible conformational changes of the fibrinogen molecules, as indicated by the fairly low clottability of the isolated fibrinogen samples. We therefore tried to elute fibrinogen with lower urea concentrations (2 M, 4 M) in the elution buffer at neutral pH. This resulted in a decrease of the amounts of fibrinogen eluted from the GlyProArgProLys-Fractogel, while the wash solution (6 M urea) contained increasing amounts of fibrinogen with the lowering of the urea concentration in the desorption buffer. Therefore the strength of binding of fibrinogen to immobilized GlyProArgProLys seems to be in the order of magnitude of the fibrin-fibrin interaction.

The alternative method recommended to desorb fibrinogen quantitatively with lower urea concentrations at acid pH is 2 M urea in 0.1 M acetate buffer pH 4.5 (suggested to us by Dr. D. Galanakis). This method is definitely superior to 6 M urea at neutral pH, since the clottability could be increased to 90% and more and this without the addition of any activated factor XIII. The efficiency of desorption of the fibrinogen could be maintained at 95% and more and the purity was greater than 95%.

Three reasons may be given for the lower clottability of the fibrinogen preparations isolated with the Gly-Pro-Arg-Pro-Lys-Fractogel affinity column as compared to fibrinogen isolated by conventional techniques. First, the presence of urea and/or acid pH may lead to some conformational changes of fibrinogen molecules most probably resulting into the formation of a weaker clot. Second, the absence of factor XIII in the fibrinogen samples may also lead to the formation of a weaker clot (see below). This clot is then disrupted to some extent by the washing procedure during the determination of the clottability. It may be partially corrected by the addition of factor XIII. Third, and probably more important, fibrinogen isolated by the affinity column is heterogeneous and contains more of the low molecular weight subfractions than fibrinogen isolated by conventional methods. It has been shown by Holm et al. (16) that the LMW fraction has a clottability of 92% and the LMW' fraction of only 80%. Since 20–23% of our Gly-Pro-Arg-binding protein is LMW- and 3–5% is LMW'-fibrinogen, it is well conceivable that this is besides the

denaturing effect of the urea the major reason for the lower clottability of the fibrinogen isolated by affinity chromatography.

Fibrinogen prepared by conventional purification procedures (ethanol or salt precipitation, e.g. Imco and Kabi fibrinogen) is more homogeneous in respect to high and low molecular weight fractions (almost exclusively fibrinogen with mw 340,000) and does not contain the low molecular weight subfractions circulating in normal blood.

Two control experiments have been performed to support the above findings. First, the treatment of Imco fibrinogen with increasing amounts of urea during several hours, then dialyzing the urea out, resulted in a considerable reduction of the clottability from 96% to 88%. Second, the observation that the clottability of fibrinogen, essentially free of factor XIII, could be gradually decreased the more intense the washing of the clot was, could be corrected to some extent by the addition of small amounts of factor XIII, indicating that the low clottability observed in our fibrinogen preparations isolated by higher urea concentrations was most probably the effect of denaturation and to some extent to the absence of factor XIII.

In order to obtain a homogeneous fibrinogen preparation with only intact fibrinogen molecules, the classical precipitation methods are still the methods of choice. With the method described here it is not possible to isolate only HMW-fibrinogen (mw 340,000 Daltons) selectively from plasma, since any catabolic intermediates containing an intact complementary binding site to GlyProArgProLys will also be retained on the column and then coeluted with the intact fibrinogen (HMW fibrinogen). On the other hand it is possible to investigate the "in vivo" fibrinogen, defined as the total clottable protein in human plasma. The GlyProArgProLys-column may also be used to quantitatively "defibrinate" plasma without activation of the coagulation cascade.

Moreover, since the amino terminal amino acid sequence of the α-chain of fibrin GlyProArg has been shown to be present in many mammals, birds and even the lamprey (5), one may expect that the complementary binding site has a similar if not identical structure in many animal fibrinogen molecules. Therefore, the method described here may also be applied to isolate fibrinogen from a vast variety of animals. Preliminary experiments have shown that the affinity isolation procedure works for rabbit and rat fibrinogen (results not shown).

Dysfibrinogens with a defective complementary polymerization site may be separated from functionally intact molecules by the use of this GlyProArgProLys-Fractogel affinity chromatography. On the other hand dysfibrinogens with α-chain polymerization site defects, which are notoriously difficult to isolate, may be quantitatively isolated from patient plasma because of their intact complementary polymerization site and they bind to the column as well as normal fibrinogen. This has been verified for fibrinogen Zürich II (results not shown).

In conclusion the GlyProArgProLys-Fractogel chromatography is a fast and quantitative method to isolate fibrinogen from plasma and is especially well suited when only small amounts of plasma are available.

Acknowledgement

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Human Fibrinogen— Structural Variants and Functional Sites

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Fibrinogen is a central protein in the blood coagulation system (1). The fibrinogen molecule is composed of three pairs of non-identical peptide chains, denoted Aα, Bβ and γ. The overall structure can thus be described as $(A\alpha, B\beta, \gamma)_2$. During blood clotting, thrombin cleaves two pairs of peptide chains, releasing fibrinopeptides A and B to form fibrin monomer with the structure $(\alpha, \beta, \gamma)_2$. The fibrin monomer can polymerize in an ordered fashion. The human fibrinogen chains Aα, Bβ and γ contain 610, 461 and 411 amino acid residues respectively (2-6) and are interconnected by 29 disulfide bridges (7-9). The covalent structure of the human protein (Fig. 1) was first elucidated by protein sequence analysis (2-5, 7-9). The work was completed in 1979 and somewhat later confirmed and extended by DNA sequence analysis (6).

Human fibrinogen occurs in a large number of different molecular forms as there are several sites or sections of the molecule which can exist as one of two or more structural alternative forms and these regional variants can combine in various ways. The number of combinations is especially large as each alternative regional form may occur on both sides, one side or neither side of the dimeric fibrinogen molecule. The regional variants can belong to either of two categories, those which are non-inherited and may be present in all individuals and those which are inherited and therefore present only in certain individuals (Table 1). The effect of the various regional molecular forms on the functions of fibrinogen is not always known.

Table 1. Regional variants

Non-inherited:	Splicing of γ-chains
	Phosphorylation of Aα-chains
	Sulfation of Bβ- and/or γ-chains
	Proline-hydroxylation of Bβ-chains
	Glycosylation of Bβ- and γ-chains
	Proteolytic degradation of Aα- and γ-chains
Inherited:	Polymorphism in Aα- and/or Bβ-chains
	Mutation in Aα-, Bβ- or γ-chain

Non-inherited variants

Three principal types of non-inherited regional variants are present in mammalian fibrinogen; they are caused by alternative splicing, posttranslational modification of specific amino acid residues or proteolytic degradation. The C-terminal region of the γ-chain occurs in two *splice-variant* forms. In human fibrinogen, the last four of the 411 residues in the more abundant form are then replaced by a stretch of 20 residues (10-12). The variants differ in that only the shortest form, which corresponds to 90% of the molecular population, can interact with platelets. However, both γ-chain forms can be cross-linked by factor XIII.

The human Aα-chain is *phosphorylated* at two serine residues (Table 2), one in fibrinopeptide A and the other in the middle part of the chain (13). It is assumed that both positions are completely phosphorylated during biosynthesis and that the phosphate groups are partially removed later by a phosphatase in the blood so that only about 20% remain. However, during an acute phase reaction, with increased synthesis giving rise to a higher level of fibrinogen, and in the fetus, up to 70% phosphorylation is observed (13). Serine phosphorylation serves as an important signal in several bio-

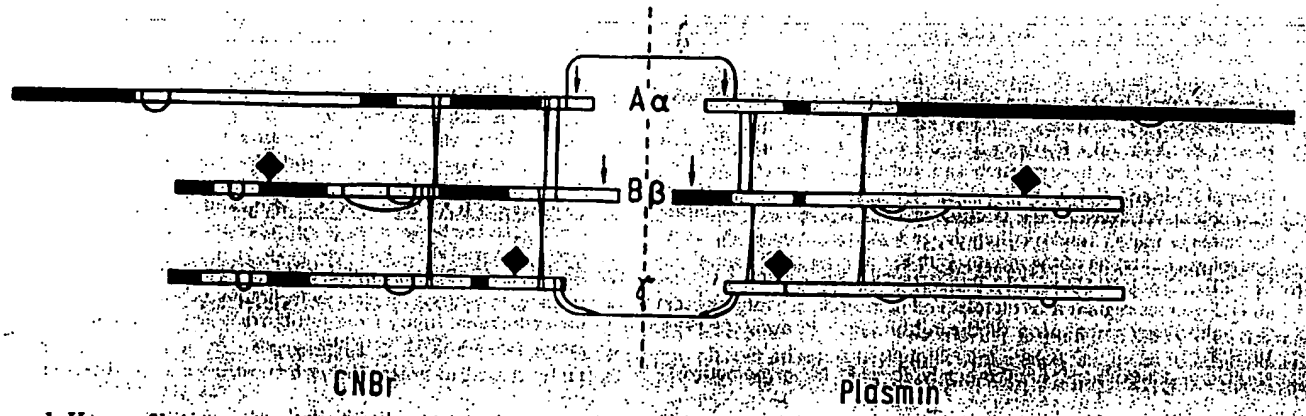

Figure 1. Human fibrinogen, a model of the covalent structure. The chains are aligned according to homology with the N-termini in the center. The thin, connecting lines represent disulfide bonds, the diamonds carbohydrate sidechains and the arrows thrombin cleavage sites. The 5 disulfide knots formed by cyanogen bromide cleavage (left) and the plasmic fragments D and E (right) are white.

Table 2. Posttranslationally modified amino acid residues

Modification	Peptide chain	Residue	Position	Sequence
Phosphorylation	Aα	S	3	ADSGEG
	Aα	S	345	NPGSSER
Sulfation	Long γ	Y	418	ETEYDSL
	Long γ	Y	422	DSLYPED
Hydroxylation	Bβ	P	31	SLRPAPP
Glycosylation	Bβ	N	364	MGENRTM
	γ	N	52	QVENKTS

logical processes, but seems to be of no functional relevance in fibrinogen, since the rate of thrombin-induced fibrinopeptide release is independent of the degree of phosphorylation.

Mammalian fibrinogen may contain *sulfated* tyrosine residues. In the human protein, only the longer γ-chain splice variant is sulfated (14). Both tyrosines in this region are fully sulfated (Table 2). The functional importance is unknown. Several animal fibrinopeptides B are tyrosine-sulfated, but not human fibrinopeptide B, as it has lost the corresponding structural region during evolution. The sulfate groups contribute to the acidic properties of the fibrinopeptides and thus to their protective effect against polymerization.

The human Bβ-chain is *hydroxylated* at a certain proline residue (Table 2) to about 20% (15). This finding was highly unexpected as hydroxyproline occurs mainly in collagen-like proteins where it is of great functional importance in regulating the optimal temperature stability of the triple helix. However, in fibrinogen the function is unknown and the sequence around the hydroxylated proline differs from the collagen hydroxylation consensus sequence.

Fibrinogen is *glycosylated* at two different sites (Fig. 1), i.e., in the N-terminal region of the γ-chain (16) and in the C-terminal region of the Bβ-chain (17). The two carbohydrate sidechains are highly similar since both are N-glycosidically linked to asparagine residues (Table 2) and are biantennary. The glycosylation at these positions is complete, but heterogeneity is caused by the presence of one or two sialic acid residues at the end of the sidechains. The amount of sialic acid influences the rate of fibrin polymerization; an increase in acidic charge delays polymerization. An increased degree of glycosylation with triantennary carbohydrate sidechains, additional sialic acid and delayed polymerization seems to occur in individuals with liver disease. A different, less specific type of glycosylation is caused by the excessive glucose level in diabetes, the glucose being bound to protein amino groups.

Proteolytic degradation affects the Aα- and γ-chains even in the blood of normal, healthy individuals. Full size, undegraded human fibrinogen has a molecular weight of 340 kDa and accounts for about 70% of the blood plasma fibrinogen. A degraded form with a molecular weight of 305 kDa accounts for 25% and one of 270 kDa for 5%. The three forms are designated as HMW, LMW and LMW', respectively, and they differ in their Aα-chains. The N-terminal region is preserved in all forms. In the LMW-form one of the two Aα-chains of the molecule is missing a C-terminal portion; in the LMW'-form both Aα-chains are C-terminally degraded (18,19). The enzyme responsible for the degradation has not yet been identified, but both plasmin and leukocyte elastase can be excluded because of their characteristic cleavage patterns (18). The heterogeneous C-terminal end of the degraded Aα-chain has been identified as positions 269, 297 and 309 (19). Differences in distribution among the HMW, LMW and LMW' forms have been observed in connection with certain diseases. Fibrin clots derived from degraded fibrinogen are less stable. An additional, minor, C-terminal degradation of about 25% of the Aα-chains leads to a variant ending at position 583, the degradation presumably being caused by plasmin as it corresponds to the earliest plasmic cleavage site. The Aα-chain is also N-terminally degraded in normal individuals, but only the first amino acid is lacking in 10% of the Aα-chains, presumably due to the action of an aminopeptidase, and this modification seems to be unimportant.

The γ-chain is also proteolytically degraded (20). The N-terminal region is completely conserved, but 6% of the γ-chains may contain only about 330 residues and 1% as little as 200 residues. The cause of the shorter γ-chains is unknown. The C-terminally degraded γ-chains are, however, unable to crosslink or to interact with platelets. A degradation form of the long γ-chain splice-variant has also been observed; it is only lacking the four most C-terminal residues (12).

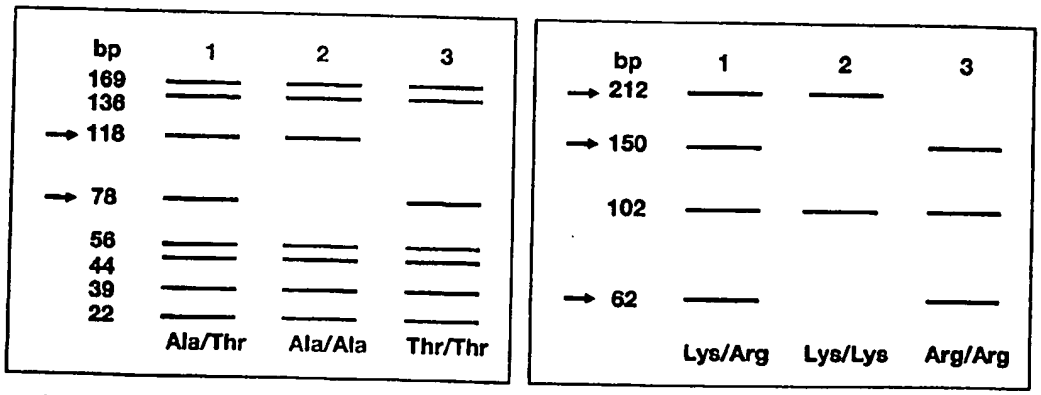

Figure 2. Polyacrylamide gel-electrophoresis patterns after amplification by polymerase chain reaction and restriction enzyme digestion characterizing the polymorphic sites in the position 312 of the Aα-chain (left) and position 448 of the Bβ-chain (right). The enzymes Rsa I and Mnl I were used with the Aα-gene and Bβ-gene product, respectively.

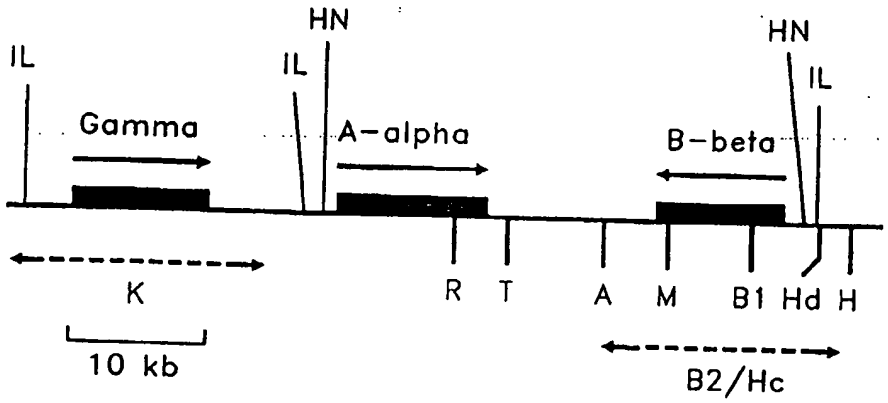

Figure 3. Fibrinogen gene region with interaction sites for HNF 1 (HN) and IL-6 (IL) and with cleavage sites for restriction enzymes Ava II (A), Bcl I (B1 and B2), Hae III (H), Hinc II (Hc), Hind III (Hd), Kpn I (K), Mnl I (M), Rsa I (R) and Taq I (T). The arrows indicate the directions of the genes and the double-arrows insufficiently characterized restriction sites.

Inherited variants

Genetically determined variants can be either common or very uncommon in a population. The common genetic variants may be detected when samples from many individuals in the population are compared. The *polymorphic* sites give rise to sequence microheterogeneity in pooled samples. The polymorphic variation is, in principle, expected to be unrelated to functional properties of the protein. A total of seven sites in human fibrinogen could be tentatively identified as polymorphic since the published protein and DNA sequence data did not agree (1). These sites, i.e., positions 47, 296 and 312 in the Aα-chain, positions 162, 296 and 448 in the Bβ-chain, and position 88 in the γ-chain, were characterized by restriction fragment length analysis after polymerase chain reaction amplification or allele-specific polymerase chain reaction amplification (21). Evidence for polymorphism was found for only two of the sites, i.e., position 312 of the Aα-chain and position 448 of the Bβ-chain (Fig. 2). An analysis of over 100 individuals in California showed the allele-frequency for the pair Thr/Ala in the Aα-chain to be 0.76 to 0.24 and for the pair Arg/Lys in the Bβ-chain 0.85 to 0.15. The polymorphism in the Bβ-chain was highly correlated with two polymorphic sites in the Bβ-gene outside the coding regions (Fig. 3), but uncorrelated with the one in the Aα-chain (22). The polymorphism in the Bβ-gene promoter region influences the interaction between nuclear proteins and the interleukin 6-response element (23) which bears upon the previously reported correlation between Bβ-gene polymorphic variants and plasma fibrinogen level and, indirectly as least, upon the property of fibrinogen as a risk factor in thromboembolic disease (24). In addition, the property of fibrinogen as a risk factor might be influenced by the highly correlated polymorphism in the coding region, placing either an Arg or a Lys residue in position 448 of the Bβ-chain.

Uncommon, inherited variants of human fibrinogen have so far only been described in association with fibrinogen dysfunctions. *Genetically abnormal* fibrinogens have now been detected in over 300 families and the structural aberrations identified in over 80 of these (1, 25). Obviously, the abnormal, dysfunctional variants can be used as highly specific probes for structure-function relationships in fibrinogen (Table 3). However, most genetic variants are discovered in hospital routine laboratories when prolonged thrombin- or reptilase-clotting

Table 3. Functional sites

Function		Detected dysfunction	Elucidated structure
Intrinsic:	Thrombin cleavage	+	+
	Polymerization	+	+
	Cross-linking	+	-
	Plasmin cleavage	+	-
Protein interaction:	Thrombin	+	-
	Factor XIII	-	-
	Plasmin(ogen)	+	-
	Plasminogen activators	+	-
	Fibronectin	+	-
	α_2-Antiplasmin	-	-
	Thrombospondin	-	-
	Albumin	-	-
	Collagen	-	-
	Lipoprotein(a)	-	-
Cell interaction:	Platelets	+	+
	Erythrocytes	+	-
	Monocytes	-	-
	Macrophages	-	-
	Endothelial cells	-	-
	Fibroblasts	-	-
	Staphylococci, streptococci	-	-
Ion binding:	Heparin	-	-
	Calcium	+	+
	Zinc	-	-
	Citrate	+	+
	EDTA	+	+

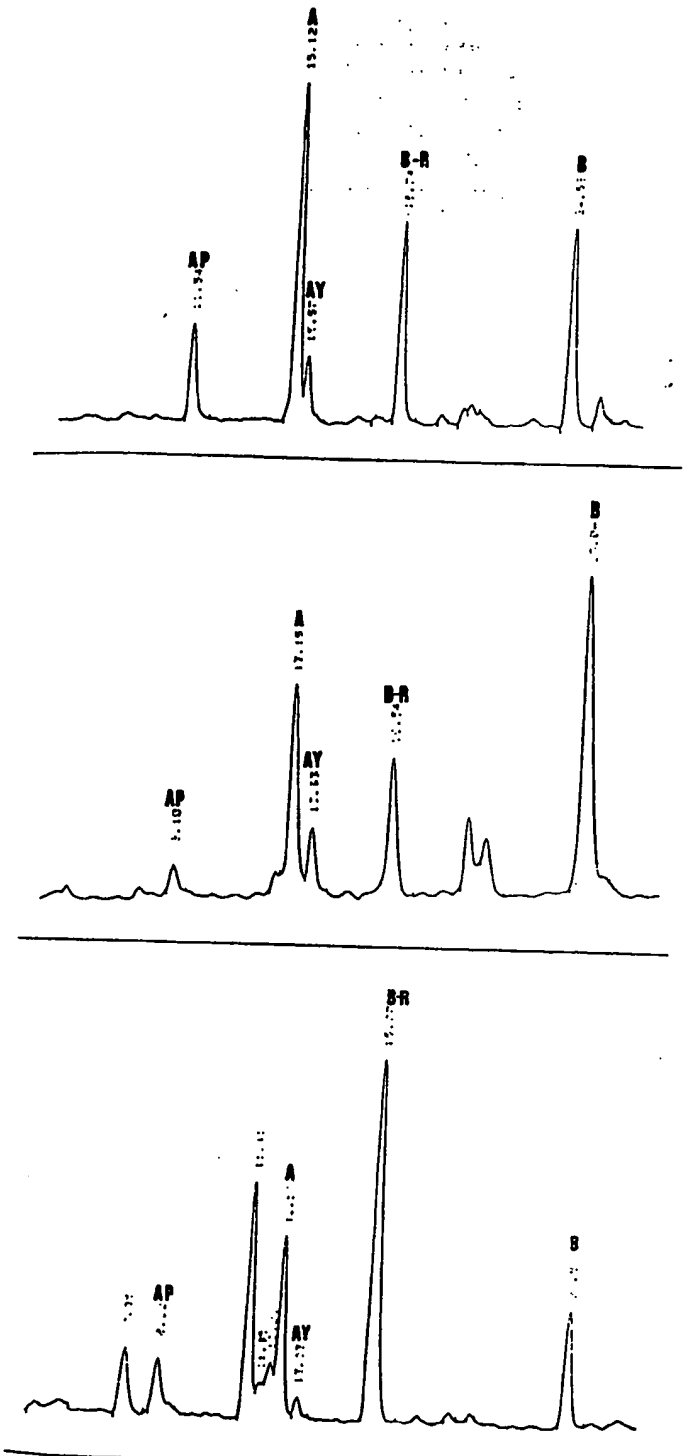

Figure 4. Reversed-phase high-performance liquid chromatography patterns of fibrinopeptides released by thrombin, characterizing fibrinogens with normal release (top), those releasing abnormal amounts of peptide as in Group IA (middle) and those releasing abnormal peptides as in Group IIA (bottom).

times are noticed. This results in a selection of those dysfunctional variants which are related to thrombin cleavage and fibrin polymerization. As a consequence, it has been useful to classify the abnormal variants by measuring the release of fibrinopeptides using reversed-phase high-performance liquid chromatography (Table 4, Fig. 4), followed by protein or, more recently, DNA sequence analysis. Out of over 80 structurally elucidated, genetically abnormal variants, 55 of the structural errors were detected in the fibrinopeptide A and B regions and the corresponding thrombin cleavage sites; 20 of them were in the primary, complementary polymerization region in the C-terminal part of the γ-chain and only six in other parts of the fibrinogen structure (1, 25). It is remarkable that only 22 of the structural errors are unique to a single family. Some variants are extremely common, the Arg to His substitution at the thrombin cleavage site of the Aα-chain being observed in 26 families, and the Arg to Cys substitution at the same site in 18 families. In both cases substitutions have a world-wide distribution, which may make common ancestry for families with the identical substitution an unlikely explanation.

The dysfunctional variants structurally elucidated so far provide some limited information about those functional properties of fibrinogen which may be unrelated to thrombin cleavage and fibrin polymerization (Table 3). However, it is to be expected that if test systems for the many additional, pathophysiologically highly relevant fibrinogen functions were developed and/or more generally employed, different and also highly informative sets of dysfunctional variants could be discovered. Several of the structurally elucidated genetic variants have supplied special, additional information about fibrinogen. Thus, the presence of a novel disulfide bridge between the two halves of the dimeric fibrinogen molecule at the level of the thrombin cleavage site in the Aα- or the Bβ-chain indicates that both Aα- and Bβ-chains are in parallel orientation at the N-terminus and that the thrombin cleavage sites of the two halves are in close proximity to each other. Furthermore, the exclusive presence of symmetrically abnormal and symmetrically normal molecules and absence of hybrid molecules in heterozygous individuals with genetic fibrinogen variants points to not yet understood protein biosynthetic mechanisms.

The complete or partial *absence* of fibrinogen from the circulating blood may be predicted to be caused by many types of genetically abnormal fibrinogen protein and/or gene structure, though it may be assumed that faults in the specific biosynthesis mechanism or chromosome structure could also be responsible for the defects. So far no detailed structural information is available for any individual lacking blood plasma fibrinogen. However, in a recent study of five unrelated families in which at least one member was completely devoid of fibrinogen in the blood, evidence was found for the presence of all three fibrinogen peptide chain genes. After amplification by polymerase chain reaction of chain-specific gene regions, products of the expected size were detected (manuscript in preparation). In several families with low levels of fibrinogen in the blood, fibrinogen was stored in large amounts in liver cells, most likely due to a protein structure abnormality which prevented proper secretion (26).

Table 4. Fibrinopeptides released by thrombin

Group	Homozygote		Heterozygote	
IA	No	FPA	1 normal	FPA
	2 normal	FPB	2 normal	FPB
IB	2 normal	FPA	2 normal	FPA
	No	FPB	1 normal	FPB
IIA	2 abnormal	FPA	1 abnormal	FPA
	2 normal	FPB	1 normal	FPA
			2 normal	FPB
IIB	2 normal	FPA	2 normal	FPA
	2 abnormal	FPB	1 abnormal	FPB
			1 normal	FPB
III	2 normal	FPA	2 normal	FPA
	2 normal	FPB	2 normal	FPB

Conclusions and perspectives

Normal human fibrinogen contains at least six regional variants in the Aα-chain, three in the Bβ-chain and five in the γ-chain. Each of these 14 regional variants may be symmetrically or unsymmetrically distributed in the fibrinogen molecules, but one or two variants may occur in genetically homozygous form (Fig. 2). From this can be calculated that each individual would carry at least 3^{12} or over half a million combinations of non-identical fibrinogen molecules in the blood. The various molecular forms may differ considerably in their functional properties, and it may be hypothesized that certain combinations of regional variants could be related to pathological conditions of the individual.

The genetically abnormal fibrinogen variants are commonly used to study structure-function relationships. It is generally assumed that the most fundamental biological role of fibrinogen lies in its ability to form the skeleton of the blood clot and thereby prevent blood leakage. For many of the functional sites related to clot formation and clot dissolution, the corresponding dysfunctional variants have indeed been identified (Table 3). However, the spectrum of genetic variants is limited because of the present procedures for discovering these variants. The wider use of screening tests aimed at detecting abnormal fibrinogens that confer increased risk of thromboembolic disease seems especially urgent. Furthermore, fibrinogen is believed to play a significant role in many additional pathophysiological processes, such as those related to wound healing, tumor growth and metastasis, and defense mechanisms. The corresponding fibrinogen dysfunctions and abnormal variants have not yet been discovered. Novel fibrinogen functions may be revealed by a study of the additional kind of diseases that unrelated individuals with identical amino acid substitution have in common. From a protein-chemical point of view, the genetically abnormal variants often seem highly similar to the variants present in normal individuals, which should suggest caution when structural data are interpreted in terms of function.

Animal fibrinogen models for structure-function relationships and for the various posttranslational modifications can provide useful information. In particular, baboon or other primate fibrinogens may serve as models for a human-like, fully functional, but still variant fibrinogen. Human and baboon fibrinogen show over 92% positional identity; the N-terminal regions are virtually indistinguishable, the middle regions of the peptide chains between the two disulfide rings account for 25% and the C-terminal regions of the Aα-chains for 65% of all differences between the two species (manuscript in preparation). The findings are reminiscent of earlier observations with more distantly related species (1). Only certain of the human regional variants have so far been detected in the baboon fibrinogen.

Acknowledgments

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QUANTITATION OF THE THREE NORMALLY OCCURRING PLASMA FIBRINOGENS IN HEALTH AND DURING SOCALLED "ACUTE PHASE" BY SDS ELECTROPHORESIS OF FIBRIN OBTAINED FROM EDTA-PLASMA.

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ABSTRACT

Normal human plasma contains fibrinogens of different molecular weight. To quantitate these fibrinogens, plasma was clotted at pH 6.4 with 7.5 NIH U/ml thrombin in the presence of 8 mM EDTA. The clots were dissolved in 6.6 M urea and submitted to SDS electrophoresis on gels containing 3% polyacrylamide/0.5% agarose, and the fractions quantitated by densitometric scanning. The reproducibility of this method was high with variation coefficient 1.5%.

Three main fibrinogen fractions were found: High molecular weight fibrinogen (HMW, mw 340 000), low molecular weight fibrinogen (LMW, mw 300 000) and LMW' (mw 280 000). In addition 5 weak bands could be seen. In plasma from 123 healthy subjects of both sexes, aged 1-93 years, HMW constituted 69.7%$\pm$5.1, LMW 26.5%$\pm$4.8 and LMW' 3.8%$\pm$1.8 of the total fibrinogen. Women and older subjects displayed a small, but statistically significant reduction in the relative amounts of HMW (2-4%).

Following surgery and extensive acute myocardial infarction the HMW/LMW ratio changed. The substantial increase in total fibrinogen regularly recorded was mainly due to HMW that reached maximal values after 3-4 days. LMW remained unchanged the first 2 days and then displayed a slight increase with a delayed maximum (8-11 days).

Key words: Plasma fibrinogen, fibrinogen heterogeneity, fibrin, SDS-gel electrophoresis

INTRODUCTION

Fibrinogen heterogeneity in normal, untreated plasma was demonstrated by SDS electrophoresis more than 10 years ago (1, 2). Such fibrinogen fractions were quantitated by densitometric scanning of the gels (2,3,4). However, as other plasma proteins than fibrinogen interferred with the scanning, the scan difference between gels loaded with plasma and with homologous serum had to be recorded (2,3). Later fibrin instead of fibrinogen has been used to exclude non-clottable proteins (5).

These methods have demonstrated that normal plasma fibrinogen contains three major fractions: High molecular weight fibrinogen (HMW) or band I fibrinogen (mw 340 000), low molecular weight fibrinogen (LMW) or band II fibrinogen (mw about 300 000) and low molecular weight fibrinogen' (LMW') or band III fibrinogen (mw about 280 000). However, the relative amounts of the fractions have varied to some extent with the method used, and the validity of the various methods is not known. Moreover, only a very limited number of samples from healthy subjects have been examined so far (6,4,5).

Finally, only few studies have dealt with fibrinogen fractions during diseases states (3,4,5,6,7). From one of these studies (3) it emerges that postoperative fibrinogen increase was entirely due to increase in HMW, no concomitant increase in LMW was recorded. This observation is difficult to reconcile with the concept that LMW is a derivative of HMW (3,7,8).

In the present study we wanted to study fibrinogen heterogeneity during so-called "acute phase". Before doing so it was necessary to establish a satisfactory method for quantitation of the fibrinogen fractions, and to state their normal range. The present study was designed to deal with these problems.

MATERIALS

Reagents:

Thrombin, bovine (Topostasine from Hoffmann-La Roche, Basel, Switzerland) was dissolved in 0.15 M NaCl to 30 NIH U/ml and stored at -20°C in polystyrene tubes.

Phosphate buffer containing EDTA. To phosphate buffer pH 6.1 (7) was added 1/40 vol Na_2-EDTA (0.5 M) to a final concentration 12.5 mmol/l.

Fibrinogen was purified from human plasma as described by Jakobsen and Kierulf (10).

Thrombo-Wellcotest, Wellcome Diagnostics, The Wellcome Foundation Limited, Dartford, England, DA1 5AH.

Soybean Trypsin Inhibitor (STI) type 1-S from Sigma Chemical co. St. Louis, Mo., USA.

Trasylol 20 000 KIU/ml from Bayer, Leverkusen, West Germany.

STI was dissolved in Trasylol and the mixture diluted with 0.15 M NaCl to STI 2.5 mg/ml and Trasylol 12.500 KIU/ml.

Sample buffer was prepared according to Weinstein and Deykin (4): TRIS-HCl buffer pH 8.0 (1 M) 2 ml, Sodium-Dodecylhydrogen-

Sulphate (SDS) 10% 20 ml, EDTA (acid form mw 292.25) 58 mg, urea 40 g, Bromphenolblue 1% 0.5 ml and H_2O to 100 ml.

Staining solution containing 1 vol. Coomassie Brilliant Blue 0.5% and 9 vol. destaining solution.

Destaining solution 1 vol. 96% ethanol, 1 vol. glacial acetic acid (100%) Merck, and 8 vol. H_2O.

METHODS

Plasma samples. Blood was collected in 5 ml test tubes containing 16.3 mmol EDTA, centrifuged at 1300 x G for 10 min at +4°C, plasma pipetted off, frozen at -20°C and examined within one week.

To study the importance of protease inhibitors, 0.1 ml STI/Trasylol mixture (see Materials) was added to the test tubes prior to sampling, giving final plasma concentrations 0.1 mg/ml and 500 KIU/ml respectively.

Fibrinogen concentration was measured according to Jacobsson (9).

Fibrinogen antigen positive material in the clot supernatant was measured by the Thrombo-Wellcotest reagents.

Preparation of gels (3% polyacrylamide/0.5% agarose) was performed using a modification (4) of the method of Fairbanks et al (9).

Preparation of fibrin. Fibrinogen was harvested by clotting in presence of EDTA giving non-crosslinked, ureasoluble clots: To 0.4 ml plasma (or purified fibrinogen) was added 0.8 ml phosphate buffer containing EDTA (see reagents) and 0.4 ml thrombin (30 NIH U/ml). After 2 hours at room temperature the clots were synerised, washed in 0.15 M NaCl, dissolved in sample buffer (see reagents), diluted to a fibrin concentration of 0.2-0.3 mg/ml and incubated at +60°C for 15 min. With this technique, occlusion of non-clottable material in the clot is negligible (9). Furthermore, less than 1% of the fibrinogen could be recovered in the clot supernatant.

SDS-electrophoresis. 25 ul (5-7.5 ug fibrin)of the samples were applied on each gel and electrophoresed for a total of 4 hours at 25V (30 min), 75V (30 min) and 125V (180 min). The gels were stained at +60°C for 30 min and appropriately destained at room temperature, at least for 3-4 days.

Quantitation of the fractions was performed by densitometric scanning using LKB Ultroscan, giving the relative amounts of the different fractions by means of a program fitted for a computor (ABC 80) which integrated the peaks.

Electrophoresis of plasma and serum. Plasma was diluted with sample buffer to fibrinogen concentration 0.2-0.3 mg/ml. Serum was prepared by clotting plasma as described above. The supernatant after syneresis of fibrin was diluted with sample buffer to the same extent as was plasma. The samples were incubated, submitted to electrophoresis and scanned as described above. Finally, the fibrinogen fractions were assayed by subtracting the serum-scan from the plasma-scan.

The normal material consisted of plasmas from 123 donors. Four age groups were examined: Children 1-10 years (n=6) without obvious disease, blood donors 20-25 years (n=31), blood donors 50-60 years (n=34) and subjects older than 70 years (n=52). The last group were inpatients from the eye department without acute or obvious serious chronic disease. All plasmas were examined blindly.

Patient material - The surgical group consisted of 20 consecutive patients (10 females and 10 males) undergoing total hip joint replacement. The mean age of the patients was 69.7±9.9 years. During the operation the patients were given 2 vol. of SAG-erythrocytes and 500 ml Dextran 70 (6%), no plasma was added.
Blood samples were drawn preoperatively, and at intervals during the 14 days following operation. In the first 10 patients repeated samples were drawn the first 12 hours postoperatively to look for early changes in total fibrinogen and HMW/LMW-ratioes.
Vacutaniner tubes containing citrate (0.11 M) were used.
The samples were examined with respect to total fibrinogen, fibrinogen fractions, gamma-globulin concentration and haematocrit (heparinized blood samples). The samples were centrifuged immediately, and plasma frozen at -20°C. All samples from one patient were examined in parallel.
Protease inhibitors (STI+Trasylol) examined in the 5 first patients, did not influence the results and were therefore omitted.
In the 5 patients suffering from acute myocardial infarction total fibrinogen and relative amounts of fibrinogen fraction (HMW/LMW/LMW') were recorded. The first samples were drawn 6-24 hours after start of chest pain. The patients were followed by daily sampling for 10-14 days.

RESULTS

Electrophoresis of diluted plasma gave 3 main fractions (Fig.1).

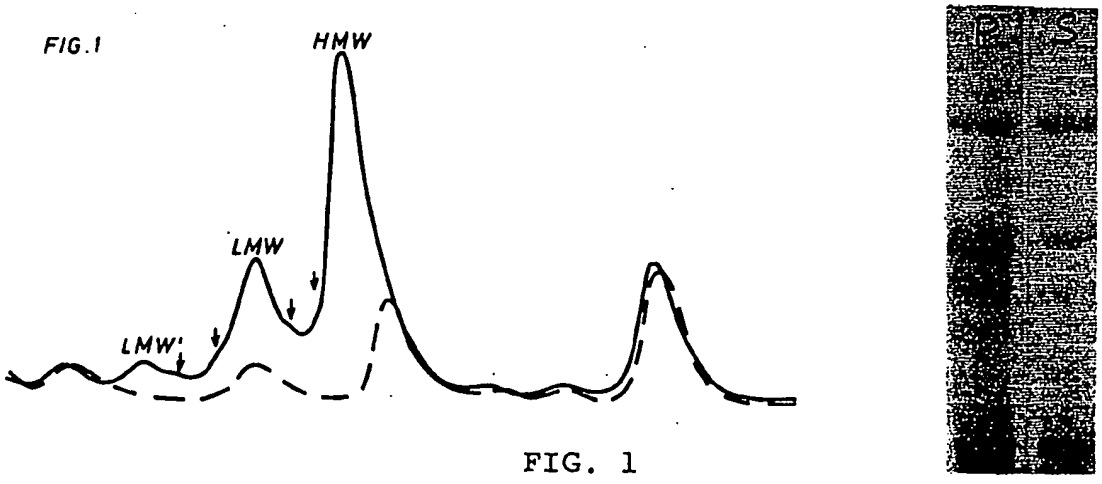

FIG. 1

Polyacrylamide gel electrophoresis and densitometric scanning of plasma (P, ——) and homologous serum (S, ----). Minor fibrinogen fractions are indicated by arrows.

In addition, 2 weak bands between LMW and LMW' were found, as were 2 bands between HMW and LMW. Two non-clottable peaks, occurring in homologous serum and interferring with scanning, had to be corrected for by subtraction (Fig.1).

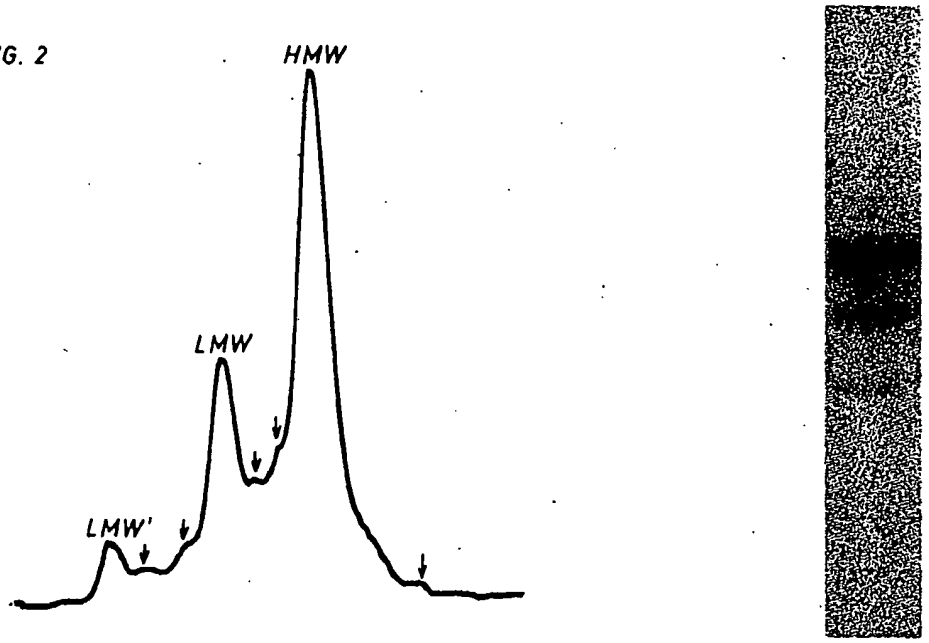

FIG. 2

Polyacrylamide gel electrophoresis and densitometric scanning of fibrin obtained from normal EDTA plasma. Minor bands are indicated by arrows.

Electrophoresis of dissolved plasma clot gave the same fibrinogen bands as in plasma (Fig. 2). Furthermore, clotting of purified fibrinogen with thrombin did not affect the relative amounts of the fractions (Table I). Finally, addition of protease inhibitors (See Methods) did not influence the electrophoretic pattern in 4 healthy subject examined in 6 parallels.

TABLE I

Effect of Clotting of Purified Fibrinogen upon the Relative Amounts of Fractions. (Mean of 4 Parallels).

	HMW	LMW	LMW'
	(% of total fibrinogen)		
Fibrinogen	67.2	29.0	3.8
Fibrin	67.8	28.5	3.7

Quantitation of the fibrinogen fractions by means of diluted plasma and serum was compared to homologous fibrin (Table II). Even if the two quantitation methods gave nearly identical mean values for HMW, LMW and LMW', it is evident that electrophoresis of dissolved clot gave more reproducible results. This method, therefore, was chosen. The variation coefficient was 1.5 within run (12 aliquots) and 3.4 between runs (10 aliquots).

TABLE II

Quantitation of Fibrinogen Fractions in 12 Aliquots of Normal Plasma by Electrophoresis of Dissolved Clot and of Diluted Plasma. The figures are % of total fibrinogen.

	Plasma	Dissolved clot
% HMW ± SD	67.8 ± 2.8	67.6 ± 1.0
% LMW ± SD	27.4 ± 1.9	28.1 ± 0.9
% LMW' ± SD	4.8 ± 1.3	4.3 ± 0.6

Distribution of fibrinogen fractions in 123 normal plasmas.

Mean and standard deviation for the three fractions (% of total fibrinogen) in the total material were as follows: HMW 69.7±5.1, LMW 26.5±4.2 and LMW' 3.8±1.9.

Relative amounts for HMW in different age groups are given in Table III. Small, but statistically significant differences ($p<0.05$) were found between the 20-25 years group and each of the two older groups. In accordance with previous observations (16), total fibrinogen increased with age.

TABLE III

Total Fibrinogen and Relative Amounts of HMW in Different Age Groups.

		HMW %		Total fibrinogen g/l
Age	no	mean±SD	range	mean±SD
<10	6	71.1	69-73	2.03
20-25	31	71.9±5.4	61-82	2.36±0.31
50-60	34	68.4±5.4	56-78	3.01±0.63
≥70	52	69.5±4.3	60-80	3.55±0.88

The women demonstrated slightly lower values for HMW in all age groups (Table IV). This difference was significant ($p<0.05$) for the total material, but not for each of the subgroups. Sex-differences in total fibrinogen was found in the two older groups with the highest values in women.

TABLE IV

Total Fibrinogen and Relative Amounts of HMW in Men and Women in Different Age Groups.

	WOMEN			MEN		
		% HMW	Fgn g/l		% HMW	Fgn g/l
Age	no	mean±SD	mean	no	mean±SD	mean
20-25	16	70.0±5.7	2.35	15	73.9±4.4	2.38
50-60	16	66.7±5.4	3.22	18	70.0±4.9	2.93
>70	29	68.8±4.3	3.84	23	70.4±4.2	3.38
Total	61	68.3±5.5	3.28	56	71.1±4.6	2.95

Fibrinogen fractions during "acute phase".

Postoperative changes in total fibrinogen, HMW and LMW (LMW+ LMW) are shown in Fig. 3. Preoperative values of fibrinogen (mean 3.56 g/l ± 0.53) and HMW (mean 67.9% ± 3.7) were in good

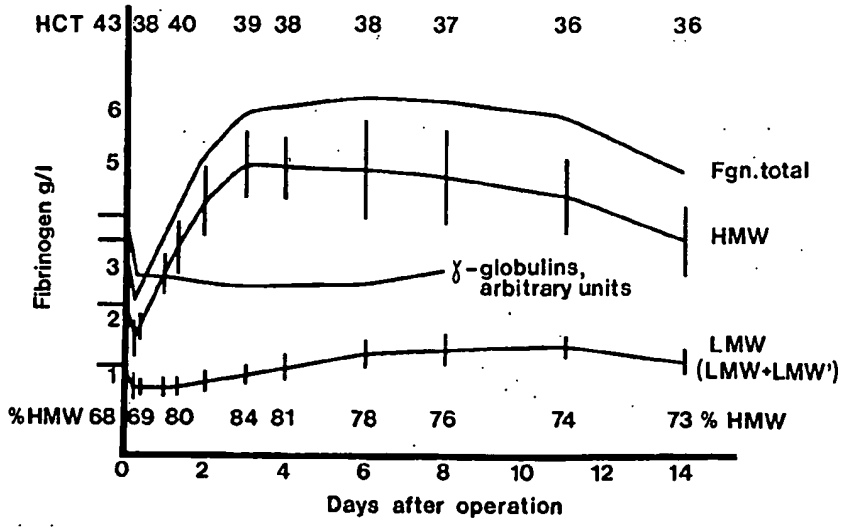

FIG. 3

Postoperative variations in total fibrinogen, HMW, LMW gamma-globulins and haematocrit in 20 patients undergoing total hip joint replacement. The figures are mean values, the bars representing 2 standard deviations.

accordance with the normal material (Table III). An initial fall in fibrinogen to 70.0% ± 8.5 of preoperative values was seen after 6 hours. A similar fall was found in gamma-globulin concentration (to 69.4% ± 3.0), but not in haematocrit.

No changes in relative amounts of the fractions (%HMW or HMW/LMW ratio) were seen until 6 (4-8) hours postoperatively when the HMW content started to rise, reaching the maximal value after 3-4 days. Total fibrinogen displayed a similar pattern with the maximal value after 5-6 days. The content of LMW, on the other hand, remained unchanged the first two days. A slight LMW-increase, first recorded after 48 hours, continued slowly reaching a maximum after 8-11 days (approximately 130% of the preoperative values.

The relative amounts of HMW (HMW/LMW ratio) started to increase approximately 6 hours postoperatively, reaching a mximum after 72 hours, and then slowly decreased. After 11-14 days the HMW/LMW ratio was nearly normalized, total fibrinogen, however, still being substantially elevated.

The described patterns were found in all patients examined. Five patients suffering from extensive acute myocardial infarction displayed similar (apart from the initial fall) changes in total fibrinogen, HMW and LMW (Fig. 4).

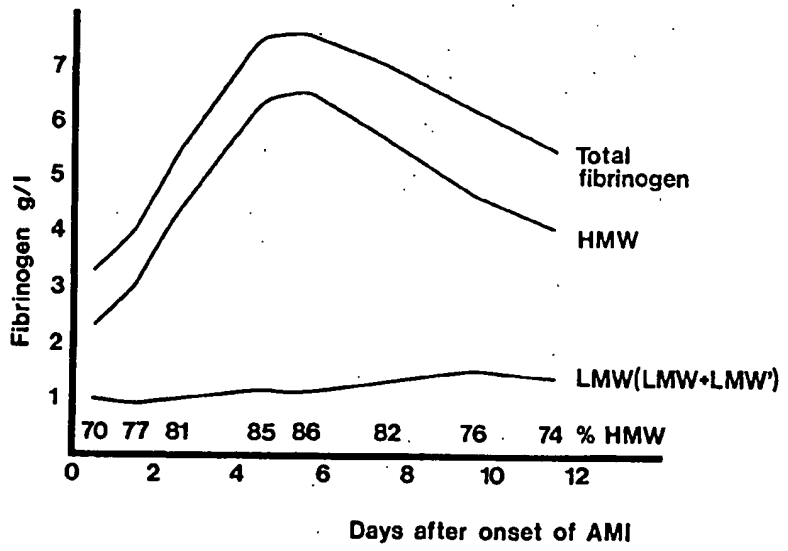

FIG. 4

Variations in total fibrinogen, HMW and LMW in 5 patients suffering from extensive acute myocardial infarction (mean values).

DISCUSSION

In the present study the optimal technical conditions for detection and quantitation of plasma fibrinogen fractions were looked for. A priori, electrophoresis of untreated plasma, containing the clottable material in its genuine form, would seem to be the method of choice. With this method three main fractions have been described (3,4,5). We have confirmed this, and in addition 4 minor bands were regularly observed, most likely due to the use of a more appropriate gel (4,13). This extensive heterogeneity corresponds well to the Aα-chain heterogeneity demonstrated by electrophoresis of reduced fibrinogen samples (4, 12,13,14,15). Unfortunately, the use of plasma is hampered by the presence of a non-clottable protein band, interferring with the densitometric scanning of HMW-fibrinogen (3).

We have confirmed this, but in contrast to previous authors (3,4) we found a second band in serum, interferring with the LMW-fibrinogen. The reason for this discrepancy is obscure, but the possibility that this second band might consist of LMW-fibrinogen not included in the clot was ruled out by the demonstration of only trace amounts of Fr.antigen positive material in the clot supernatant. The presence of non-clottable proteins in plasma, interferring with the quantitation of fibrinogen fractions, renders untreated plasma less suitable for the purpose. Accordingly, the reproducibility was poor, mainly due to technical problems associated with the subtraction of the serum-scan from the plasma-scan.

To avoid these problems, fibrin instead of fibrinogen has been used (5). As shown above in purified fibrinogen as well as in plasma, identical patterns were obtained with the two methods, but the reproducibility of the fibrin method was superior. In addition, better separation and more distinct bands were obtained. It must therefore, be concluded that electrophoresis of dissolved clots is a reliable and convenient method for detection and quantitation of fibrinogens of different molecular weight.

With this method, the mean values of the fibrinogen fractions in plasma from healthy subjects corresponded well to those obtained previously with a similar technique (5), whereas higher values for LMW fibrinogen has been found in untreated plasma (3). The present observation of a non-fibrinogen protein with electrophoretic mobility identical to that of LMW-fibrinogen, may contribute to explain this discrepancy.

In the present material, statistically higher amounts of LMW-fibrinogen (lower HMW/LMW ratioes) were found in the two older groups, as well as in women. No satisfactory explanation for these differences can be given at present.

To study changes in fibrinogen fractions during "acute phase" patients undergoing major surgery, and patients suffering from extensive acute myocardial infarction were selected. These conditions with well defined starting points, displayed significant fibrinogen-increase of limited duration. An unexpected initial fall in fibrinogen in the surgical group proved to be due to bleeding/haemodilution, as evidenced by similar falls in gamma-globulin and fibronectin concentration. The lack of con-

comitant fall in haematocrit was due to transfusion of erythrocytes (without plasma).

In both groups the increase of fibrinogen was mainly due to production of HMW. During the first 48 hours HMW seemed to increase at a nearly constant rate, 0.06 mg/ml/hr ± 0.014, examined in the surgical group. In contrast to Lipinska (3) we also found a slight increase in the LMW fraction, first recorded after 48 hours and with a very late maximum (8-11 days). The maximal values of HMW and total fibrinogen on the other hand, were recorded after 3-4 days and 5-6 days, respectively.

Apparently the major part of fibrinogen is synthesised as HMW. The possibility than even LMW is synthesised as such cannot be excluded from the present study, but like others (7,8, 12) we find it most likely that LMW is a derivative of HMW. This transformation, however, must be a rather slow prosess, perhaps requiring some age of the HMW-molecules before the -COOH terminal end(s) of the Aa-chain(s) (4,14) can be proteolysed. Furthermore, presuming a half life of LMW comparable to HMW (8,17), we find our observations in accordance with the theory that only a minor part of HMW is metabolised to LMW (8). In spite of the agreement that LMW is a derivative of HMW, nothing is known about the mechanism of this prosess.

Changing HMW/LMW ratio during acute phase reaction is a consequence of the described variations in HMW and LMW. This ratio was unchanged the first few hours, and started to increase 4-8 hours after start of operation. The first week was characterised by abnormally high relative amounts of HMW (%HMW), the ratio was gradually normalized during the second week, total fibrinogen still being substantially elevated.

The properties of HMW and LMW are different with respect to solubility, thrombin clotting time and polymerization rate (17,18). Probably the normal ratio represents an optimal balance between fractions of different functional properties. The possibility that the described changes in fibrinogen quality during acute phase might be of functional importance has to be considered. Thus, in a previous study we found variations in the amounts of the heparin precipitable fraction of plasma that might be due to variations in fibrinogen quality (19). Regarding possible in vivo consequences of changes in fibrinogen quality, further studies are needed.

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